Running head: The algal Rubisco micro-compartment

Title: The Algal Pyrenoid: Key Unanswered Questions

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Abstract

The confinement of Rubisco into a chloroplast micro-compartment, or pyrenoid, is a distinctive feature of many micro-algae, and possibly contributes to around 30 Pg of carbon fixed each year. Our understanding of pyrenoid composition, regulation, and function, however, are still fragmentary. The model pyrenoid of *Chlamydomonas reinhardtii* is increasingly well-resolved under different regimes of light or inorganic carbon availability. The emergence of molecular details in other lineages provides a comparative framework for this review, and evidence that most pyrenoids function similarly, even in the absence of a common ancestry. The objective of this review is to explore pyrenoid diversity throughout key algal lineages and discuss whether common ultra-structural and cellular features are indicative of common functional processes. By characterising pyrenoid origins in terms of mechanistic and structural parallels, we hope to provide key unanswered questions which will inform future research directions.

Part A: Form Follows Function - Compartmentalisation Requirements For Operating A CO_2-Concentrating Mechanism In A Single Cell

Pyrenoids are permeable Rubisco-containing micro-compartments present in the chloroplast stroma of many, but not all, algae operating a biophysical CO_2-concentrating mechanism (CCM) (Badger *et al*., 1998; Raven, 2010). CCMs enhance the CO_2 concentration near the primary carboxylating enzyme, Rubisco, through the coordinated action of membranal inorganic carbon (Ci) pumps, one or more carbonic anhydrases (CA), and generally, the packaging of Rubisco into one, or multiple pyrenoids. Common in unicellular, colonial or filamentous algae with examples in nearly all lineages except Chrysophytes (Lee, 2008; Maberly *et al*., 2009), pyrenoids are rarer in frond-forming seaweeds (pyrenoid-positive examples include the sea lettuce *Ulva* or the edible red alga *Pophyra*). The prokaryotic analogues of pyrenoids, carboxysomes, are an obligatory feature of cyanobacterial CCMs (reviewed in Raven *et al*., present issue), whereas pyrenoids are not obligatory. When present, pyrenoids physically separate the site of CO_2-fixation from C_i accumulation by the CCM machinery (primarily thought to be via plasmamembrane and chloroplast envelope, and perhaps direct delivery to the pyrenoid). Such an aggregation of Rubisco enhances CCM effectiveness, as demonstrated empirically through quantification of CCM-leakiness, and loss of C_i accumulation affinity, when Rubisco is redistributed to the stroma in *Chlamydomonas* mutants (Meyer *et al.*, 2012).

The advantages of a pyrenoid were initially quantified in unicellular green algae through demonstrations that the concentration of internal C_i was 5-10X higher in pyrenoid possessing algae than phylogenetically close species lacking the chloroplastic micro-compartment (Morita *et al*., 1998). Pyrenoids can therefore be viewed as an evolutionary adaptation enhancing the performance of a basal CCM consisting only of C_i pumps and

CA(s), within the constraints of a single cell, without the need for multicellular specialisation. The absence of a C_i impermeable boundary or shell around the pyrenoid nevertheless imposes an additional compartmentalisation requirement: where to localise the CA that dehydrates C_i to CO_2 such that leakage is minimised? Current models posit its localisation either to the lumen of trans-pyrenoidal thylakoids or at the pyrenoid periphery. The seminal experiment by Price and Badger (1989) demonstrating that in cyanobacteria, a CA must be packaged alongside Rubisco into the micro-compartment to avoid short-circuiting the CCM, has yet to be validated in eukaryotic algae.

The Rubisco matrix can be either naked or enclosed by starch plates forming a sheath, together with peri-pyrenoidal protein complexes or parts of the chloroplast envelope, with likely effects on CO_2 permeability. Membranes can provide a conduit between the stromal pool of C_i and the heart of the pyrenoid, but C_i entry could also occur *via* proximal diffusion or other channels. These peripheral elements, when present, should probably be viewed as defining features of pyrenoids. The first part of this review will integrate recent developments with information from the historical literature to update our understanding of the three major pyrenoid components - a Rubisco matrix (common to all pyrenoids), thylakoid lamellae traversing the matrix, and peripheral elements – and conclude with models on how these could interact.

Composition And Inner Architecture Of The Pyrenoid Matrix

The word pyrenoid (from the Greek *pyrene*, stone or kernel-like) was coined by Schmitz (1882) to describe highly refractive near-spherical inclusions in algal chloroplasts examined through a light microscope. Schmitz observed pyrenoids in the majority of green algae he studied, to a lesser extent in red algae and only occasionally in brown algae. The generalised use of transmission electron microscopy (TEM) from the early 1950s onward greatly

facilitated the diagnosis of pyrenoid presence/absence, as the matrix of these micro-compartments appears as uniformly electron-dense inclusions in the stroma. Arguably, it was only with the advent of techniques capable of discerning Rubisco localisation (*e.g.* immuno-gold labelling, indirect-immunofluorescence tagging or translational fusions with fluorescent proteins) that pyrenoids were incontrovertibly established as the site of Rubisco localisation (see Fig. 1).

Early biochemical analysis of pyrenoids isolated from green and brown algae found that ~90% of the matrix was composed of biochemically active Rubisco, alongside a dozen or so other unidentified proteins (reviewed in Meyer & Griffiths, 2013). For green algae, this included the chaperone Rubisco Activase, where localisation to the matrix was confirmed by immuno-cytochemistry (McKay & Gibbs, 1991a; Suess *et al*., 1995). Non-green algae do not code for Rubisco Activase, but express a CbbX protein instead (reviewed in Kroth, 2015), which belongs to an unrelated AAA+ ATPase gene family with an Activase-like property (Mueller-Cajar *et al*., 2011). Whether CbbX also localises to non-green pyrenoids remains, as yet, unknown. Pyrenoid compositional analysis was refined by mass spectrometry for *Chlamydomonas* (Mackinder *et al*., 2016), which in addition to Rubisco and Rubisco Activase, identified EPYC1 (formerly known as LCI5, Miura *et al*., 2004). EPYC1 was particularly abundant in pyrenoids isolated from cells acclimated to CCM-active conditions (*i.e.* grown under air-level CO_2) and is speculated to act as a linker that either recruits Rubisco to the matrix or serves as an anchoring scaffold for the enzyme. Genes coding for proteins with properties similar to those of EPYC1 are present in other pyrenoid-positive algae (*e.g.* diatoms and haptophytes) (Mackinder *et al*., 2016).

It is still unclear to what extent, if at all, Rubisco is arranged periodically within the matrix. Resin embedding for TEM usually obliterates the native arrangement of the enzyme, leaving pyrenoids to appear as amorphous. There have been, however, several reports of

107 para-crystalline structures of the matrix or a fraction thereof, *e.g.* in Chlorophytes (Bertagnoli
108 & Nadakavukaren, 1970) and Charophytes (Gärtner & Ingolić, 1989), in diatoms
109 (Holdsworth, 1968; Taylor, 1972), in dinoflagellates (Kowallik, 1969), in Haptophytes
110 (Leadbeater & Manton, 1971), and in red algae (McBride & Cole, 1972; Tsekos *et al*., 1996).
111 These early studies concluded a possible cubic- or hexagonal-closed packing. Hexagonal-
112 closed packing was also identified in a recent study of the fine architecture of the
113 *Chlamydomonas* pyrenoid, using techniques that preserve the native molecular conformation,
114 by Engel and co-workers (2015). Although the analysis was limited to small areas of the
115 matrix and the alignment was not perfectly crystalline, it fitted models of periodically
116 arranged Rubisco linked directly by EPYC1 (Mackinder *et* al., 2016).
117 Encouraged by early successes in isolating pyrenoids, comparative MS studies of
118 pyrenoid composition should now be undertaken on key representatives of all major
119 phytoplankton lineages, to identify additional components helping to aggregate Rubisco and
120 other factors that are commonly present. Understanding the fine mechanistic details will also
121 require crystallographic reconstructions of protein-protein interactions and validation through
122 the characterisation of mutants, which is now increasingly possible in the model system
123 *Chlamydomonas*, for which insertional mutant libraries covering more than 80% of all coding
124 genes are available (Li *et al*., 2016).
125
126 *Function Of Pyrenoidal Tubules And Association With Carbonic Anhydrases*
127 Most, but not all pyrenoids appear to be traversed by at least one lipid bilayer, usually, but
128 not always, in continuity with the stromal network of thylakoids. These membranes are
129 assumed to play a role in the delivery of CO_2 to Rubisco. The complexity of the membrane
130 network traversing the Rubisco matrix, when present, has been used as a taxonomic marker
131 (*e.g.* in dinoflagellates, Dodge, 1968, or diatoms, Schmid, 2001). Fig. 2 illustrates the

diversity and complexity of this feature, as it appears in thin TEM sections, throughout key algal lineages. The simplest is in the form of a single membrane bisecting the Rubisco matrix, as in many *Chlorella* species (Ikeda & Takeda, 1995; see also recent example in Treves *et al*., 2016) or diatoms (Schmid, 2001). Multiple, non-connecting parallel membranes, are common in green algae, dinoflagellates and Euglena (Kusel-Fetzmann, 2008). More complex morphologies have been observed in the unicellular red alga *Porphyridium cruentum*, where a highly anastomosed network increases the surface area in contact with Rubisco (McKay & Gibbs, 1991a). The pattern is somewhat reminiscent of the one found in the green alga Zygnema, which computational 3D reconstructions revealed to match a gyroid cubic organisation of photosynthetic membranes (Zhan *et al*., 2017). The *Chlamydomonas* pyrenoid is structurally the best resolved, following the work by Engel and colleagues (2015), building on earlier studies (Sager & Palade, 1957; Ohad *et al*., 1967). Here, pyrenoid-specific membranes are formed by the fusion of stromal thylakoids into cylindrical membranes ~100 nm across, called tubules. These tubules are continuous with stromal thylakoids. When extending into the pyrenoid matrix, tubules twist and turn at sharp angles to fuse into an interconnected star-shaped network, or knot, at the pyrenoid centre (see SI animation). Additionally, tubules contain within their lumen between two and eight mini-tubules, formed "outside-in" as the thylakoid membranes coalesce. As a result, mini-tubules enclose their own lumenal phase, which is continuous with the chloroplast stroma. Mini-tubule dimensions are sufficient for the transit of small molecules like Rubisco substrates and products but too small for the shuttling of larger proteins, say a CA. There is no evidence yet that trans-pyrenoid membranes in other algae also possess similar inner channels. We speculate that the central star-shaped knot of tubules of the *Chlamydomonas* pyrenoid could play a role in situating and anchoring the Rubisco matrix in a conserved chloroplastic locus.

156 Two lines of evidences support the notion that tubules may serve a similar function
157 across a wide range of algal lineages. The first pertains to a CCM critical CA: in
158 *Chlamydomonas* and in the marine diatom *Phaeodactylum tricornutum*, this CA is localised
159 to the lumen of tubules (Karlsson *et al.*, 1998; Blanco-Rivero *et al.*, 2012; Kikutani *et al.*,
160 2016). It is pivotal to the functioning of the CCM (mutants have a high-CO_2 requiring
161 phenotype) but sets an additional requirement for a C_i transporter without which the stromal
162 pool of C_i could not be fed to the lumenal CA for conversion to CO_2. Chlorella also has a CA
163 associated with trans-pyrenoidal thylakoids (Villarejo *et al*., 1998), but whether it is lumenal
164 or even essential to the CCM has yet to be demonstrated. A second line of evidence is the
165 biochemical nature of pyrenoidal tubules, which is distinct from stromal thylakoids. O_2-
166 evolving Photosystem II (PSII) are absent from these membranes in green algae (McKay &
167 Gibbs 1991a), red algae (Mustardy *et al*., 1990) and diatoms (Pyszniak & Gibbs, 1992). This
168 is maybe an evolutionary adaptation to minimise oxygen production in the vicinity of
169 Rubisco, and hence potential for oxygenation, but the mechanism by which PSII is excluded
170 has yet to be investigated. Light harvesting antennae of PSII and their accessory pigments are
171 also excluded from pyrenoids, as shown by localisation experiments of phycobilisomes and
172 phycoerythrin in red algae (McKay and Gibbs, 1990a; Tsekos *et al*., 1996).
173 Universality of the above arrangement is challenged by tubule-less pyrenoids. Stalked
174 pyrenoids that bulge from the chloroplast into the cytosol in a sac-like structure are frequently
175 not traversed by membranes. In these pyrenoids, the Rubisco matrix is almost fully enclosed
176 by the chloroplast envelope, and in species with a secondary or higher order chloroplast, by
177 additional lipid-bilayers. Tubule-less pyrenoids are common in Phaeophytes, dinoflagellates
178 (Dodge, 1973), and Chlorarachniophytes (Ishida *et al*., 1999). In most instances however,
179 part of the Rubisco matrix is at least tangentially in contact with stromal thylakoids. Tubule-
180 less pyrenoids are also observed to a limited extent in red algae, green algae and diatoms. If

we discount the possibility that published micrographs simply failed to capture rare membranes, it will be important to localise the closest CA, and determine whether Ci accumulation is stromal in these species. Part of the answer will also come from better imaging of pyrenoids, either through increased use of 3D sectioning and reconstruction microscopy (*e.g.* focused ion beam or serial block face scanning-electron microscopy; see SI animation) or through confocal imaging of pyrenoid-specific proteins.

Pyrenoid Matrix Peripheral Structures

The Rubisco matrix of green algal pyrenoids is often surrounded either partially, or almost entirely, by starch. Red algae and algae that inherited a red algal chloroplast through secondary endosymbiosis can also have their pyrenoid encased by starch, but only when it is stalked (Ford, 1984). This can easily be explained by differences in site of starch synthesis and deposition: it is stromal in “greens” but cytosolic in “reds”. The close spatial relationship between starch and pyrenoids, even when situated in different cellular compartments, is perhaps indicative of a positive role for carbohydrate deposition in the CCM. In green algae, starch formation around the pyrenoid is controlled by light and the state of CCM induction (Kuchitsu *et al*., 1988; Ramazanov *et al*., 1994; Lin & Carpenter, 1997; Borkhsenious *et al*., 1998), which in turn also determine the maximal packaging of Rubisco to the pyrenoid matrix (Mitchell *et al*., 2014; Tirumani *et al*., 2014). A starchless Chlorella mutant with naked pyrenoid has been used to question the role of the starch sheath in the CCM (del Pino Plumed *al*., 1996), but Chlamydomonas mutants with partial or no starch sheath have a high-CO_2 requiring phenotype (Thyssen *et al*., 2003). There is therefore a pressing need to clarify the relationship of starch and the CCM and to further investigate the distinct nature of pyrenoidal and stromal starch granules (Izumo *et al*., 2007).

Calvin Benson Basham Cycle (CBBC) enzymes other than Rubisco are absent from the Chlamydomonas pyrenoid matrix (Suess *et al.*, 1995). McKay & Gibbs (1991b) found phosphoribulose kinase (PRK), which operates just upstream of Rubisco, in stromal inclusions of pyrenoid tubules (which may in fact represent mini-tubules *sensu* Engel *et al*., 2015), and proposed that this provided a means for exchanging CBBC metabolites between pyrenoid and stroma. However, PRK forms a dimer of >70 kDa, which is well above the estimated size-exclusion of mini-tubules, qualifying the localisation of this enzyme to the pyrenoid. The co-purification of Fructose-1,6-bisphosphatase with Chlamydomonas pyrenoids and their starch sheath (in SI Mackinder *et al*., 2016) suggests that the CBBC may nevertheless operate in close proximity to the pyrenoid. Identifying the CBBC location in relation to the starch synthesis pathway would also clarify the role of the different starch forms in algae with chloroplastic starch.

Finally, evidence is emerging in Chlamydomonas that there is yet another layer to pyrenoids, in the form of a network of proteinaceous complexes residing outside the starch sheath (~440 kDa, encoded by two genes, *lcib* and *lcic*). Crystallisation of the two monomers, as well as the finding of a functional homologue in the diatom *P. tricornutum* (Jin *et al*., 2016), confirmed that these proteins had a typical CA fold, although no CA activity was found in Chlamydomonas. It therefore remains open to debate what purpose this complex serves. A true CA in the stroma, as mentioned above, would short-circuit the CCM. It is tempting to speculate that LCIB-LCIC, subject to tight regulation, could be active only when CO_2 concentrations are in excess of other C_i species, and the CA-moiety operates uni-directionally from CO_2 to bicarbonate, acting to recapture CO_2 leaking from the pyrenoid. Immuno-gold labelling of LCIB and LCIC revealed deposition in pockets rather than forming a continuous ring around the pyrenoid (Yamano *et al*., 2010) and it must be clarified whether these coincide with the starch plate interfaces and thylakoid tubule entry points, where

leakage of CO_2 is likely to be maximal. Alternatively, the complex could serve as a non-catalytic structural barrier, or even play a positional role in situating the pyrenoid in the chloroplast, as suggested by pyrenoid-mislocalisation phenotypes in mutants with aberrant localisation of LCIB (Yamano *et al*., 2014).

Part B: Pyrenoid Plasticity And Dynamics Across Cell Divisions

In addition to the diversity across different algal species highlighted above, the pyrenoid is also highly plastic, changing in terms of morphology and composition in response to different cues, both endogenous and externally derived. This section focuses on the former, and specifically assesses the way in which pyrenoid morphology, structure and composition change as a function of cell-cycle progression. Additionally, the way in which the pyrenoid is accommodated through the process of cytokinesis and cell division is also considered. At all stages, considerations are not restricted to the model alga *Chlamydomonas reinhardtii*, and a wide range of algal species are used to assess the existence of commonalities, and inform evolutionary considerations.

Pyrenoid Dynamics And The Cell Cycle

Progression through the cell cycle necessitates significant changes to the physiology and functioning of an algal cell, given the extensive preparations that must be undertaken before division can successfully occur. Many of these significant changes, and their effect on the pyrenoid, are often lost to studies using asynchronous cell populations, and thus many of the associations between cell cycle stage and pyrenoid/CCM functionality remain under-explored. This section will consider how the composition and structure of the pyrenoid changes as the cell progresses through the stages of the cell cycle, and specifically during cytokinesis and the act of cell division.

Previous studies assessing the activity of the CCM over time have shown it to vary with the cell cycle (Sültemeyer, 1997) but whether this is due to variation in pyrenoid function or the activity and abundance of other CCM components remains outstanding. Considerations of different algal species have produced contrasting results. The localisation of Rubisco to the pyrenoid during the cell cycle of the green alga *Dunaliella tertiolecta*, suggest that aggregation of Rubisco was independent of cell cycle stage, and was more likely to be associated with the active growth phase (Lin & Carpenter, 1997). By contrast, results observed in the brown alga *Scytosiphon lomentaria* highlighted the formation of new Rubisco aggregates separate from existing pyrenoids during mid-S phase (Nagasato *et al.*, 2003). Disruption of the cell cycle using specific pharmacological agents further clarified the relationship between changes in pyrenoid morphology and specific cell cycle events: blocking DNA replication using aphidicolin inhibited the formation of new pyrenoids, whereas the disruption and blocking of the process of mitosis using nocodazole resulted in an increased size of Rubisco aggregates compared to untreated cells. Addition of chloramphenicol resulted in no new occurrence of pyrenoids, despite the successful completion of mitosis and cytokinesis, suggesting that these aggregates were a product of newly-synthesised Rubisco.

This study points to the role of distinct cellular events in shaping pyrenoid composition, morphology and structure during specific cell cycle events. Overall however, there is a relative paucity of data analysing the impact of the cell cycle on pyrenoid dynamics, and the efforts that are present throughout the literature have been restricted to a limited number of algal species. More work is needed to assess the effect of cell cycle on pyrenoid structure and composition, and by extension, CCM function. There is also a need to characterise such putative cell cycle dependencies at a molecular level - recent evidence suggests that as much as 80% of the Chlamydomonas transcriptome displays a strong

periodicity in cells where the cycle has been synchronized under a standard dark-light cycle (Zones *et al.*, 2015). Thus studies linking the ultrastructural changes observed to cell cycle dependent changes in transcriptional output would be highly instructive in furthering our understanding of the processes driving pyrenoidal dynamics throughout the cell cycle.

Pyrenoid Dynamics During Cell Division

Pyrenoid morphology and dynamics have been explored extensively during the process of mitotic cell division. Such a cell division necessitates the equable distribution of parental contents to daughter cells and poses problems for cells whether containing a single pyrenoid or multiple pyrenoids. Griffiths (1970) broadly divided pyrenoid containing algae into two groups based on the behaviour of the pyrenoid during the process of mitosis. One group encompassed species wherein the pyrenoid divides either prior to or concomitant with the cell division, whereas the other consisted of algae where the pyrenoid disappears during division, and reforms *de novo* in daughter cells. There is perhaps one other possibility not considered by Griffiths, which is potential *de novo* pyrenoid formation in parental cells, followed by distribution to daughter cells. Surveys of the available literature provide supporting evidence for the existence of each of these processes, in different algal species, and will be explored below.

De Novo Pyrenoid Formation - Before And After Cell Division

One of the first reports utilising electron microscopy to study the pyrenoid examined the green colonial alga *Scenedesmus quadricauda* and identified the disappearance of the pyrenoid in parental cells, followed by reappearance in daughter cells, suggesting dissolution and *de novo* formation (Fig. 3A) (Bisalputra & Weier, 1964), a phenomenon supported by more contemporary reports (Vítová *et al.*, 2008). Experiments conducted in another green

alga, *Tetracystis excentrica* obtained similar results, with “regression and dissolution” of the pyrenoid prior to cell division observed (Brown & Arnott, 1970). A similar phenomenon was also observed in *Euglena gracilis*, which possesses a secondary chloroplast, with disappearance of the pyrenoid prior to cell division, followed by reformation in daughter cells (Osafune *et al.*, 1990). Other algae appear to lack the dissolution mechanism - in *Volvulina steinii* a single daughter cell inherits the parental pyrenoid, implying *de novo* formation following cell division in the other (Fig. 3B) (Nozaki *et al.*, 1987). Other species, such as *Scytosiphon lomentaria* form a second pyrenoid *de novo* in the parental chloroplast prior to cell division, with the two pyrenoids now present in the chloroplast then being equally distributed among daughter cells upon division (Fig. 3C) (Nagasato & Motomura, 2002). Whilst the observation of *de novo* pyrenoid formation across a range of algal species supports the existence of this mechanism as a means of ensuring pyrenoidal continuity across cell divisions, there is some ambiguity surrounding the exact nature of the bodies forming *de novo* in some of these strains. In her landmark 1970 study, Goodenough observed a number of dense bodies that superficially appear similar to the pyrenoid, although ultimately discounted the notion that they might represent new pyrenoids. Irrespective, it is apparent that if such a premise of *de novo* pyrenoid formation is correct, it inevitably raises numerous questions, perhaps most notably with regards to the location in which they form. Specifically, why do they form there and are there any features of that particular location, ultrastructural or otherwise, that are permissive, conducive or essential to pyrenoid formation? Addressing such questions through comprehensive studies of a diversity of different algae will allow physical and structural features of the chloroplast to be correlated with *de novo* pyrenoid formation and the processes underpinning biogenesis.

Pyrenoid Fission

The process of pyrenoid fission during mitosis is comparatively well established. Electron microscopy based experiments in Chlamydomonas established that both the pyrenoid and chloroplast in this algal species divide by fission (Fig. 3D) (Goodenough, 1970). In this study, a marked increase in pyrenoidal mass prior to cell division was observed, concomitant with a lateral elongation perpendicular to the plane of the furrow driving chloroplast fission; subsequent narrowing of the furrow and further elongation ultimately results in a roughly even partitioning of the pyrenoid, and the formation of two daughter pyrenoids from the original parent. This phenomenon has similarly been observed in a wide array of different algal species, including *Porphyridium cruentum* (Gantt & Conti, 1965), *Porphyridium purpureum* (Schornstein & Scott, 1982), *Pleurochrysis haptonemofera* (Hori & Inouye, 1981) and *Isochrysis galbana* (Hori & Green, 1985). In addition to these red algae and haptophytes, division of the pyrenoid in this way has also been observed and confirmed in the brown algal species *Cylindrocapsa germinella* (Sluiman, 2004), as well as *Splachnidium rugosum* and *Scytothamnus australis* (Tanaka et *al.*, 2007). Thus, the process of fission and its role in ensuring equitable distribution of pyrenoids appears to be a common phenomenon present across many evolutionarily distinct clades of pyrenoid possessing algae. In context of this apparent conservation of pyrenoidal fission across a diverse range of algal strains, an interesting question arises from consideration of the morphological diversity in body plans that exists among these species - whereas some are polarised (as for *Chlamydomonas reinhardtii*), others (such as *Porphyridium purpureum*) are radially symmetric, and thus, despite conservation of the process by which pyrenoidal division occurs, the mechanisms underpinning such divisions might be differentially regulated.

FtsZ And A Role For the Ancestral Contractile Machinery?

354 The question remains however as to how exactly such a process might occur: increasing
355 evidence highlights a role for plastid division proteins such as FtsZ. Originally descended
356 from cyanobacterial cell division proteins (Miyagishima & Kabeya, 2010), the GTPase FtsZ
357 assembles into a ring-like structure on the stromal surface of the chloroplast prior to division.
358 Through a poorly understood mechanism, FtsZ, along with components on the cytoplasmic
359 face of the chloroplast, then generates the contractile force required for membrane
360 constriction, and eventually, fission (Osawa *et al.*, 2008). Recent work by Hirakawa *et al.*
361 demonstrated the function of FtsZ proteins in the secondary plastid of chlorarachniophytes,
362 and in *Bigelowiella natans*, both FtsZD-1 and FtsZD-2 formed a ring-like structure that
363 bisected the midpoint of a bilobate pyrenoid found in the secondary chloroplast of this
364 species (Hirakawa & Ishida, 2015). This ring was constitutively present at this region and
365 was associated with a shallow furrow that penetrated the pyrenoid. Intriguingly, qPCR
366 analysis of gene expression was not suggestive of an involvement with the actual act of
367 plastid division *per se*, instead being upregulated following cell division, perhaps suggesting
368 a role in determining pyrenoid positioning following establishment of the daughter
369 chloroplast.
370 The study highlights the role that FtsZ proteins have in affecting pyrenoid
371 morphology. Such results are further supported by studies of algae possessing primary
372 plastids, namely *Scenedesmus quadricauda* where immuno-electron microscopic approaches
373 identified FtsZ structures localised around pyrenoids (Vítová *et al*., 2008). Whilst it was
374 unclear whether such structures were rings or a hitherto unobserved spherical arrangement, it
375 highlighted the existence of FtsZ proteins not directly associated with the stromal
376 chloroplastic membrane, and is suggestive of a functional role for these proteins in pyrenoid
377 morphology, possibly coordinating the division of the chloroplast with the pyrenoid.
378 Intriguingly, Vitova *et al.* noted that FtsZ levels did not differ between untreated cells and

cells in which DNA replication had been inhibited. Thus, control of activity, rather than expression levels, might be the key factor delimiting FtsZ activity to specific stages of the cell cycle. The question remains however as to how FtsZ activity might be temporally delimited to the period immediately leading up to cell division.. There is significant evidence that phosphorylation has the capacity to affect the functionality of structural components involved in cell and plastidial division. Phosphorylation has long been known to reversibly control the localisation of various microtubule associated proteins with the actin cytoskeleton in the cytoplasm (Ozer & Halpain, 2000). Phosphorylation modulates interactions between bacterial cell division components, notably FtsZ and FipA (Sureka *et al.*, 2010) in mycobacteria. The strong conservation of these components across both algal and prokaryotic lineages suggests that in similar systems, activity of FtsZ can be modulated by phosphorylation. Previous work had highlighted the strong dependence of the phosphorylation state of thylakoid proteins on the stage of the cell cycle in Chlamydomonas (Marcus *et al.*, 1986), thus raising the possibility that dynamic and reversible chemical modification of FtsZ might play a role in delimiting its mechanical effects on pyrenoid morphology to specific periods of the cell cycle.

Evolutionary Routes To Diversity In Accommodating The Pyrenoid

It appears that there exists substantial diversity between algal species as to how the pyrenoid is accommodated during the process of cell division. Mounting evidence suggests a functional role for the cyanobacterially derived family of FtsZ proteins and indeed, their ancestral nature is consistent with the observation of pyrenoidal fission across a wide range of evolutionary disparate algal species. The existence of others modes of ensuring successful propagation of pyrenoids to daughter cells raises intriguing possibilities - the fact that some plastid division proteins are depleted in certain algal species (Miyagishima *et al.*, 2014) raises

the question as to whether loss of FtsZ might have prompted the diversification of pyrenoid accommodation strategies during cell division from fission to other methods, such as dissolution followed by *de novo* formation. Why this loss might occur is unclear, and indeed, it would perhaps be considered disadvantageous given the evident capacity for such a system to automatically couple pyrenoid division to plastid and cellular division. Analyses examining the presence or absence of particular FtsZ proteins across different algal strains employing different pyrenoid division processes would be particularly timely, allowing differences in presence/absence to be related to the phenotype observed.

Conclusion – Integrating pyrenoid composition and dynamics

Notwithstanding independent origins, that will be clarified when the detailed molecular compositions can be compared across algal lineages, pyrenoids appear to deliver saturating concentrations of CO_2 to Rubisco on a limited set of functional elements. Identifying interactions within the Rubisco matrix and between the matrix and tubules and peripheral elements should now be a major priority for the CCM research community. Fig. 4 illustrates three possible modes of high-level pyrenoid biogenesis and regulation. Interacting inter-dependencies (Fig. 4a) depicts a model in which major component are under independent genetic control. The recruitment of Rubisco to and the situation of the matrix within the chloroplast requires a tightly regulated interplay between all three components. Proof of concept could be the finding of mutants which retain tubules and/or pyrenoid-peripheral elements in a locus where the pyrenoid would normally form, even when the Rubisco matrix is lost. Hierarchical or "Russian nesting doll" model (Fig. 4b) assumes a unidirectional and sequential formation (and dispersion when the CCM is repressed), starting with the deposition of Rubisco around a conserved anchoring site, with secondary deposition of peripheral elements. The hybrid model (Fig. 4c) integrates the two previous ones and

429 accounts in particular for the observation that a CO_2-acclimation independent fraction of
430 Rubisco is always retained in the pyrenoid (as in *Chlamydomonas reinhardtii*, Borkhsenious
431 *et al.*, 1998; Mitchell *et al*., 2014). Simpler models can be derived for naked pyrenoids and
432 pyrenoids not surrounded by peripheral elements.

433 Though now published over 50 years ago, Bisapultra and Weier were correct in their
434 declaration that 'to understand the function of such organelles as the pyrenoid, developmental
435 studies are necessary' (Bisalputra & Weier, 1964). Though perhaps not completely correct in
436 their categorisation of the pyrenoid, the notion they put forth is as timely then as it is now. To
437 truly comprehend the dynamic, malleable structure that is the pyrenoid, and by extension, its
438 role and place in the existing CCM paradigm, approaches that explore the variability in this
439 sub-cellular micro-compartment, both across the cell cycle and across a range of algal species
440 are required.

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Figure Legends

Figure 1: Pyrenoid diagnostic in *Chlamydomonas reinhardtii*

(a) The pyrenoid matrix and surrounding starch sheath are easily identifiable in unicellular green algae, as illustrated by the model alga *Chlamydomonas*, using only light microscopy (here, enhanced with Nomarski interference contrast). **(b)** In electron microscopy, the pyrenoid appears as electron dense matrix traversed by trans-pyrenoidal thylakoids (tubules), surrounded by slightly spaced starch plates, indicating that the carbohydrate deposition does not fully encapsulate the Rubisco matrix. **(c,d)** Definitive proof of preferential Rubisco targeting to the pyrenoid requires additional methods, like electron microscopy of immunogold-labelled Rubisco (c) or confocal imaging of fluorophore-tagged Rubisco (d). [All images by MTM; transformational plasmid used in (d) as per Mackinder *et al*., 2016]

Figure 2: Examples of morphological diversity of micro-algal pyrenoid matrix and associated network of tubules

(a) Green algae, with examples taken from Cladophorales and Siphonocladales (after Hori & Ueda, 1975), and *Chlamydomonas reinhardtii* ("star shaped" tubules); (b) Red algae (after Gantt & Conti, 1965; Ford, 1984); (c) Chlorarachniophytes (after Ishida *et al*., 1999); (d) Diatoms (after Bedoshvili *et al*., 2009); (e) Dinoflagellates (after Dodge, 1973). Legend: **dots** = pyrenoid matrix (mainly composed of Rubisco); **thick lines** = stromal thylakoids when outside the pyrenoid matrix or trans-pyrenoidal thylakoids (tubules) when traversing the pyrenoid matrix; **hatched boxes** = peri-pyrenoidal starch plates, stromal in green algae and cytosolic in non-green algae; **dashed lines** = membranal delimitation between chloroplast

and cytosol.

Figure 3: Speculative models of pyrenoid biogenesis integrating all three major components.

(a) Interacting inter-dependencies depicts a model in which major component are under independent genetic control. The recruitment of Rubisco to and the situation of the matrix within the chloroplast requires a tightly regulated interplay between all three components. Proof of concept could be the finding of mutants which retain tubules and/or pyrenoid-peripheral elements in a locus where the pyrenoid would normally form, even when the Rubisco matrix is lost. **(b)** Hierarchical or "Russian nesting doll" model assumes a unidirectional and sequential formation (and dispersion when the CCM is repressed), starting with the deposition of Rubisco around a conserved anchoring site, with secondary deposition of peripheral elements. **(c)** The hybrid model integrates the two previous ones and accounts in particular for the observation that a CO_2-acclimation independent fraction of Rubisco is always retained in the pyrenoid (as in *Chlamydomonas reinhardtii*, Borkhsenious *et al.*, 1998; Mitchell *et al*., 2014). Note that simpler models can be derived for naked pyrenoids and pyrenoids not surrounded by peripheral elements.

Figure 4: The diversity of mechanisms in different algal species ensuring pyrenoidal continuity across mitotic cell divisions.

(a) Apparent dissolution of the pyrenoid in the parent cell, followed by *de novo* formation in each daughter cell upon cytokinesis and completion of cell division e.g. *Scenedesmus quadricauda*. **(b)** Inheritance of the pyrenoid by a single daughter cell, with *de novo* pyrenoid formation in the other e.g. *Volvulina steinii*. **(c)** *De novo* pyrenoid formation preceding

cytokinesis and cell division, with the two pyrenoids in the parent cell then distributed equally between daughter cells e.g. *Scytosiphon lomentaria*. **(d)** Fission of the parental pyrenoid leading to its equitable distribution between daughter cells e.g. *C. reinhardtii*.

Figure 1

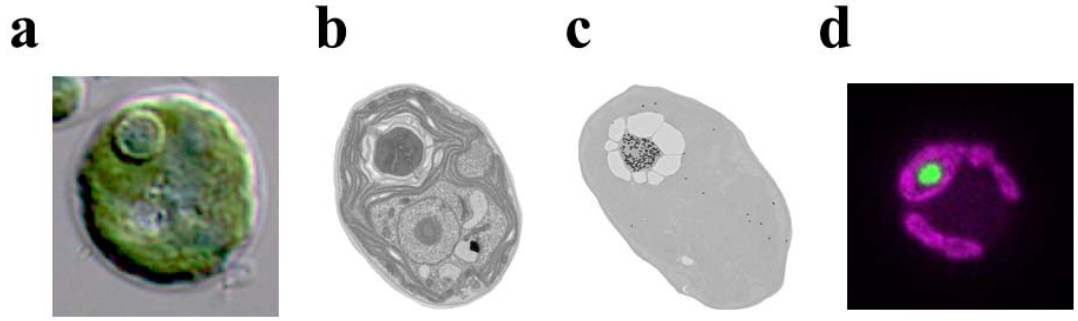


Figure 2

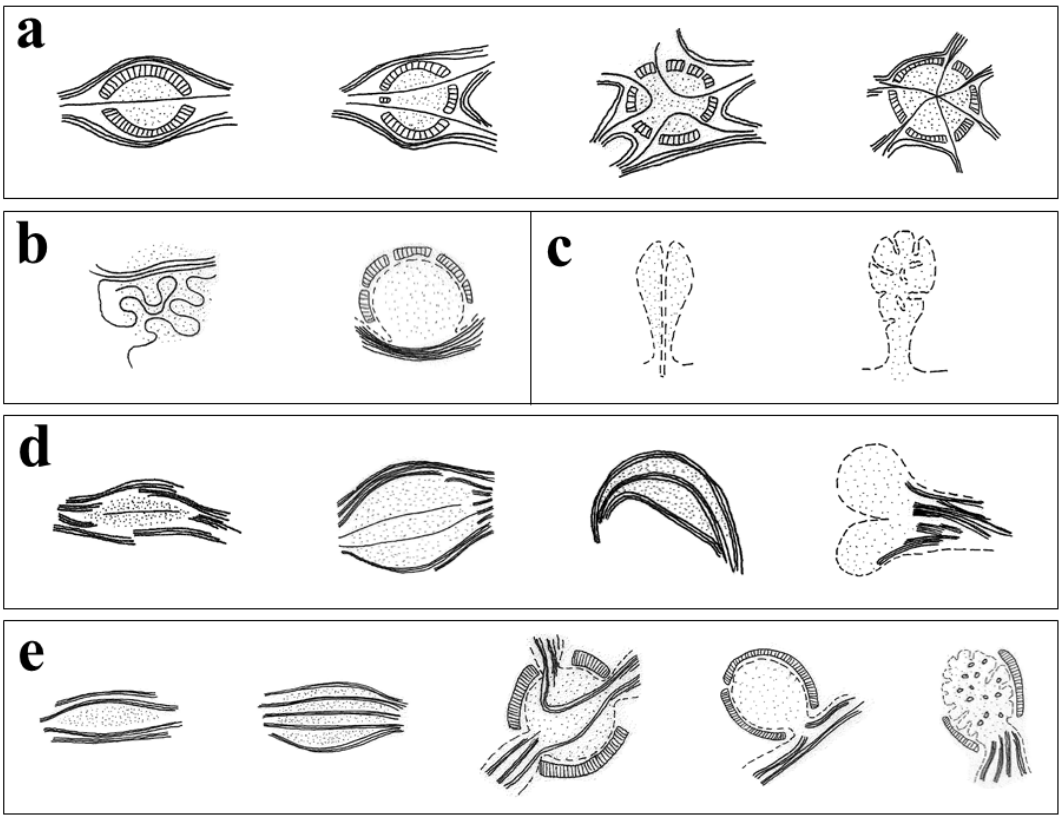


Figure 3

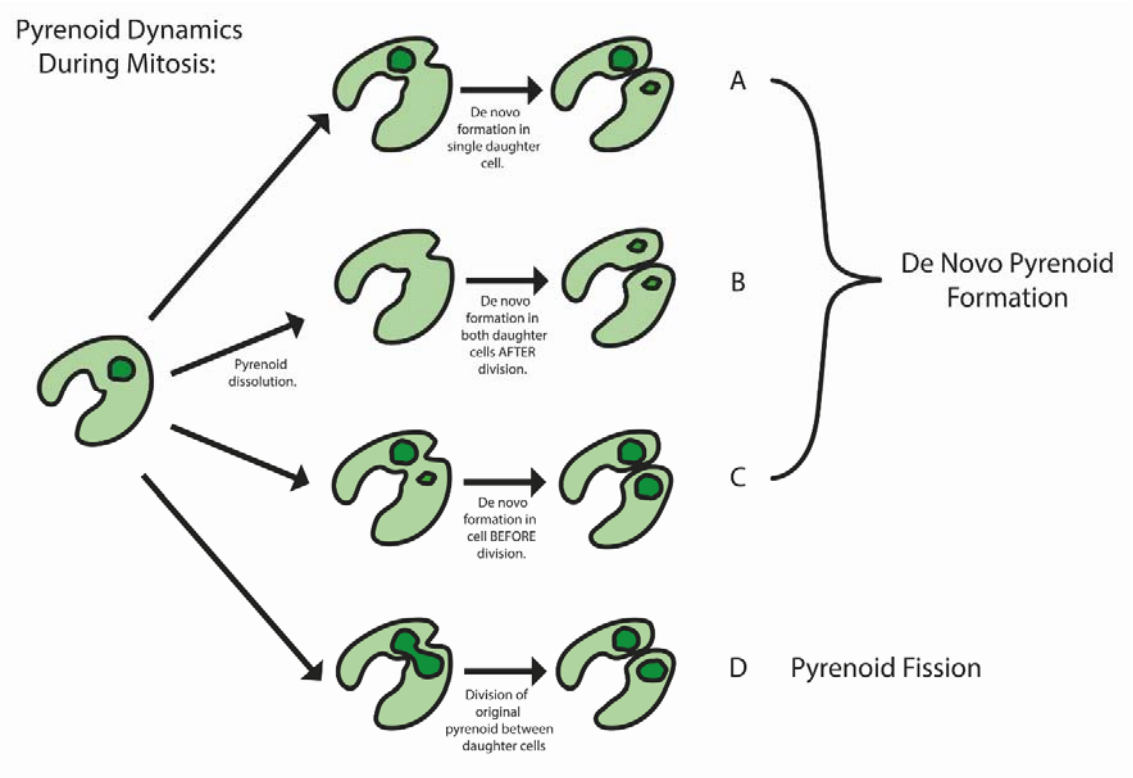
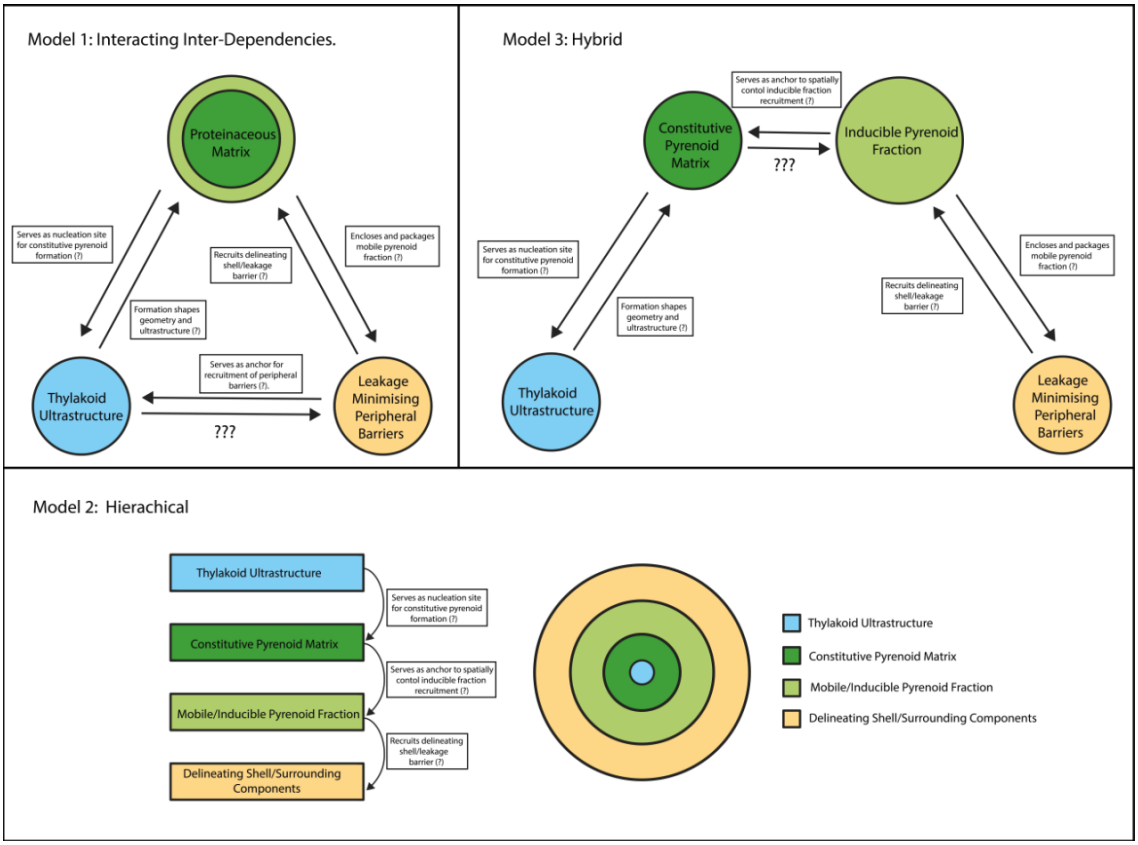

Pyrenoid Dynamics
During Mitosis:
Pyrenoid
dissolution.
De novo
formation in
single daughter
cell.
De novo
formation in
both daughter
cells AFTER
division.
De novo
formation in
cell BEFORE
division.
Division of
original
pyrenoid between
daughter cells
A
B
C
D
De Novo Pyrenoid
Formation
Pyrenoid Fission

Figure 4


Model 1: Interacting Inter-Dependencies.
Proteinaceous Matrix
Serves as nucleation site for constitutive pyrenoid formation (?)
Recruits delineating shell/leakage barrier (?)
Encloses and packages mobile pyrenoid fraction (?)
Formation shapes geometry and ultrastructure (?)
Serves as anchor for recruitment of peripheral barriers (?).
Thylakoid Ultrastructure
Leakage Minimising Peripheral Barriers
???
Model 3: Hybrid
Serves as anchor to spatially contol inducible fraction recruitment (?)
Constitutive Pyrenoid Matrix
Inducible Pyrenoid Fraction
???
Serves as nucleation site for constitutive pyrenoid formation (?)
Formation shapes geometry and ultrastructure (?)
Recruits delineating shell/leakage barrier (?)
Encloses and packages mobile pyrenoid fraction (?)
Thylakoid Ultrastructure
Leakage Minimising Peripheral Barriers
Model 2: Hierachical
Thylakoid Ultrastructure
Serves as nucleation site for constitutive pyrenoid formation (?)
Constitutive Pyrenoid Matrix
Serves as anchor to spatially contol inducible fraction recruitment (?)
Mobile/Inducible Pyrenoid Fraction
Recruits delineating shell/leakage barrier (?)
Delineating Shell/Surrounding Components
Thylakoid Ultrastructure
Constitutive Pyrenoid Matrix
Mobile/Inducible Pyrenoid Fraction
Delineating Shell/Surrounding Components